REAPPRAISAL OF "BENIGN" LYMPHOEPITHELIAL SIALADENITIS FOR EVIDENCE OF EXTRANODAL MARGINAL ZONE B-CELL LYMPHOMA

by

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MASTER'S THESIS

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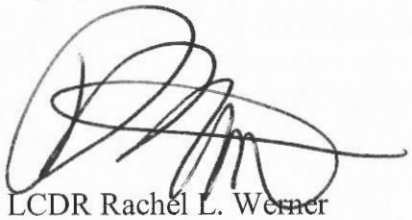

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ABSTRACT

Reappraisal of "Benign" Lymphoepithelial Sialadenitis for Evidence of Extranodal Marginal Zone B-cell Lymphoma

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Introduction. Since the earliest descriptions of lymphoid lesions in the salivary glands, there has been debate regarding the differentiation of reactive and neoplastic lesions, benign and malignant processes and the significance of molecular features such as light chain restriction or immunoglobulin (Ig) heavy chain gene rearrangement. Lymphoepithelial sialadenitis (LESA), formerly known as benign lymphoepithelial lesion (BLEL), is a reactive process characterized by the infiltration of lymphocytes into the salivary gland. Extranodal marginal zone B-cell lymphoma (EMZBCL) is a malignant lymphoproliferative disease thought to arise in mucosa - associated lymphoid tissue (MALT) acquired in the development of LESA. Differentiating between these lesions is challenging due to their morphologic similarities, although modern molecular and immunohistochemical (IHC) staining techniques may provide mechanisms to reliably distinguish them.

Objective. The aim of this study was to evaluate archival historic cases of BLEL for morphologic features, immunohistochemical profile, evidence of monoclonality and the presence of cytogenetic alterations previously identified in EMZBCL.

Methods. Twenty cases of BLEL involving major salivary glands (18 parotid and 2 submandibular) were retrieved from the Joint Pathology Center Tissue Repository and evaluated

for morphologic, immunophenotypic, molecular and cytogenetic abnormalities associated with EMZBCL.

Results. Cases comprised 19 female patients and 1 male patient, ages 16-78 years (median age 47). All cases displayed lymphoepithelial lesion formation with epitheliotropic monocytoid lymphocytes. Fifteen cases demonstrated monoclonal heavy chain gene rearrangements by polymerase chain reaction (PCR), seventeen cases demonstrated kappa light chain restriction, eight cases demonstrated increased copy number of chromosome 3 via fluorescence in situ hybridization (FISH) and eleven cases demonstrated increased copy number of chromosome 18 via FISH. No cases revealed translocations involving the MALT1 gene. Epitheliotropic B cells exhibited a CD20, CD79a and PAX 5 positive B-cell immunophenotype; aberrant CD43 expression was present in 17 cases and 2 cases demonstrated aberrant CD5 expression.

Conclusion. A significant portion of historic cases classified as BLEL demonstrate features indistinguishable from EMZBCL based on current diagnostic criteria, including morphology, heavy chain rearrangement and cytogenetic alterations.

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I. REVIEW OF THE LITERATURE

Lymphoepithelial sialadenitis (LESA), formerly known as benign lymphoepithelial lesion (BLEL), is a chronic inflammatory process characterized by the infiltration of lymphocytes into the salivary gland. LESA is most commonly identified in patients with Sjögren's Syndrome, who typically present with bilateral swelling of the parotid glands. Extranodal marginal zone B-cell lymphoma (EMZBCL) usually arises in mucosa associated lymphoid tissue (MALT) that is acquired secondary to LESA (Abbondanzo, 2001) and differentiating between these lesions has presented a challenge because of their morphologic similarity. Modern molecular and immunohistochemical (IHC) staining techniques have provided mechanisms to reliably distinguish them. Over time, a variety of terms and eponyms have been used to describe the aforementioned bilateral parotid swelling, each with its own pathologic and prognostic implications.

The diagnosis of "Mikulicz's disease" was historically applied to the clinical presentation of bilateral lacrimal or salivary gland swelling. This term was based on the work of Johann von Mikulicz-Radecki, who, in 1892, reported a 42-year old farmer suffering from extensive bilateral swellings of the lacrimal and salivary glands (Ihrler et al. 2005). In 1952, Godwin published an article in *Cancer* describing the morphologic similarities in a series of 11 cases previously diagnosed as either chronic inflammation or Mikulicz's disease. Previous reports had described this process as neoplastic in nature and characterized the lesions as "adenolymphoma", "lymphosarcoma", or "lymphoepithelioma". Godwin instead proposed the name "benign lymphoepithelial lesion". He found this term more appropriate, as it did not commit the pathophysiologic change to a purely neoplastic or inflammatory process. Of the 11 cases

reviewed, 2 had recurred at the same site following simple excision and were subsequently treated successfully with radiation (Godwin, 1952). As a result Godwin stressed the possibility that recurring lesions represented a leukemia or lymphoma, and cautioned that a definitive diagnosis is made prior to initiating any form of treatment. Azzopardi and Evans (1971) described a relationship between Mikulicz's disease and parotid gland lymphoma, either consecutively or simultaneously, noting the diagnostic challenges presented by morphologic similarities between the two conditions. A similar report in 1976 detailed four patients diagnosed with primary lymphoma of the parotid gland that followed an indolent clinical course (Nime et al. 1976). In 1983 Isaacson and Wright proposed the term "MALT lymphoma" for this type of lymphoma (Isaacson et al. 1983). They reported a series of patients of British and Middle Eastern decent who presented with similar clinical features and a B-cell neoplasm characterized by a diffuse plasma cell infiltrate in the lamina propria of the small intestine. The following year, a second article by these authors described a morphologically identical B-cell neoplasm arising in stomach, salivary gland, thyroid and lung (Isaacson et al. 1984). They proposed that these neoplasms shared similar pathologic and clinical features with the previously described MALT lymphoma of the small intestine, including a tendency to remain localized for long periods, B-cells surrounding and invading glandular tissue to form lymphoepithelial lesions (LELs) and origin from mucosa-associated lymphoid tissue (Isaacson et al. 1984). A chronology of the terminology for lymphoid proliferation of the salivary glands is found in Table 1.

Table 1: Evolution of Terminology for Lymphoid Proliferations of the Salivary Glands			
Benign			
Authors	**Year Published**	**Terminology**	**Author's Description**
Mikulicz-Radecki (21)	1892	Mikulicz Disease	"Uniform infiltrate of small round cells with scattered acini"
Godwin (3)	1952	Benign Lymphoepithelial Lesion (BLEL)	"Mass of lymphoid tissue with scattered foci of epithelial cells of ductal origin"
Schmid, Helbron, Lennert (10)	1982	Myoepithelial Sialadenitis	"Myoepithelial proliferation and lymphoid infiltration"
Harris (10)	1999	Lymphoepithelial Sialadenitis (LESA)	"Infiltration of ductal epithelium by lymphocytes of marginal zone or monocytoid B-cell type, forming lymphoepithelial lesions"
Malignant			
Authors	**Year Published**	**Terminology**	**Author's Description**
Nime, Cooper, Eggleston (5)	1976	Primary malignant lymphomas of the salivary glands / Mixed histiocytic-lymphocytic lymphoma	"lymphomatous infiltrate showing poorly differentiated lymphocytes and malignant histiocyte-like cells" "Occasional epi-myoepithelial island [was observed] in the midst of the malignant lymphoma"
Isaacson, Wright (7)	1984	MALT-derived lymphoma	"The majority of the tumor [is] constituted by a mixture of lymphocytes and plasma cells, [which] did not invade and destroy epithelial tissues"…"lymphoepithelial lesion[s]…composed of salivary gland ducts…with accompanying epithelial proliferation" "Larger sheets of FCCS [or, follicle center cells] present, usually centered around a lymphoepithelial lesion"
Sheibani, Burke, Swartz, Nademanee, Wineberg (20)	1988	Monocytoid B-cell lymphoma	"A diffuse lymphocytic infiltrate through the glands was associated with numerous germinal centers. The lymphocytes surrounding the germinal centers appeared to be typical reactive mantel zone cells. The overall impression was that of a benign lymphoepithelial lesion; however, encircling the epi-myoepithelial island there was a monotonous infiltrate composed of neoplastic MBL. The MBL were concentrated in this area and focally infiltrated individual residual acini"
International Lymphoma Study Group (8)	1994	Marginal Zone B Cell Lymphoma, extranodal, MALT-type	"Marginal zone B-cell lymphoma is characterized by cellular heterogeneity, including marginal zone (centrocyte-like) cells (small, atypical cells resembling small cleaved follicular center cells or centrocytes, but with more abundant cytoplasm, similar to Peyer's patch, mesenteric nodal, or splenic marginal zone cells), monocytoid B cells, small lymphocytes, and plasma cells. Occasional large cells (centroblast- or immunoblast-like) are present in most cases. Reactive follicles are usually present, with the neoplastic marginal zone or monocytoid B cells occupying the marginal zone and or the interfollicular region; occasional follicles may contain an excess of marginal zone or monocytoid cells, giving them a neoplastic appearance (follicular colonization). In epithelial tissues, the marginal zone cells typically infiltrate the epithelium, forming so-called lymphoepithelial lesions."
World Health Organization (17)	2008	Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT-lymphoma)	"The lymphoma cells infiltrate around reactive B-cell follicles, external to a preserved follicle mantle, in a marginal zone distribution and spread out to form larger confluent areas which eventually overrun some of most of the follicles." "Lymphoepithelial lesions are aggregates of three or more marginal zone cells with distortion or destruction of the epithelium, often together with eosinophilic degeneration of epithelial cells."

The lymphoid proliferation that Godwin designated "benign lymphoepithelial lesion" is known in modern literature as lymphoepithelial sialadenitis, or LESA (Harris et al, 1994) and is characterized by infiltration of lymphocytes into the salivary gland, accompanied by ductal epithelium proliferation, resulting in lymphoepithelial lesion formation (previously referred to as "epi-myoepithelial islands"). LELs are characterized by hyperplastic, irregular islands of polygonal to spindled transformed ductal cells infiltrated by epitheliotropic monocytoid B-lymphocytes with concurrent loss of luminal space. In the initial stages of LESA, the degree of lymphocytic infiltration is variable. With disease progression, there is effacement of glandular structures by increasingly dense lymphoid tissue (Ellis, 2007).

LESA is most commonly identified in Sjögren's Syndrome (SS) although not all patients with LESA manifest the clinical and laboratory features of SS (Ellis et al. 2007). LESA, as well as SS, affect women in a 3 to 1 ratio, commonly present in the 4th to 7th decades of life and preferentially involve the parotid glands (90%) relative to the submandibular glands (10-15%) (Ellis et al. 2007). It is a chronic autoimmune disease in which salivary and lacrimal glands undergo irreversible damage by chronic inflammation cells, resulting in xerostomia and keratoconjunctivitis sicca. Sjögren's belongs to a spectrum of autoimmune diseases involving the inappropriate development of antibodies against connective tissue DNA. Specific to this disease are the development of anti SS-A and anti SS-B antibodies, although rheumatoid factor and salivary duct antibodies may also be present (Ellis et al. 2007). It is well-documented that SS patients have an increased risk of developing non-Hodgkin's lymphoma (Kassan et al. 1978), specifically EMZBCL of parotid gland (Ferry, 2008); however all cases of LESA do not necessarily progress to EMZBCL (Kassan et al. 1978).

Table 2: Comparison of LESA and EMZBCL of Parotid Gland		
	LESA	*EMZBCL*
Demographics	• 3:1 Ratio women to men • 30-60 years old	• Women more common than men • Average age: 63 years
Morphology	• T lymphocyte-predominant infiltrate with foci of marginal zone B-lymphocytes • Germinal center formation • Lymphoepithelial lesions composed of polygonal and spindled ductal epithelium with deposits of eosinophilic hyaline material	• Slightly enlarged B-cells with marginal zone or monocytoid appearance • Proliferation in sheets and halos around lymphoepithelial lesions • Non-neoplastic infiltrate of T lymphocytes in intrafollicular areas • Plasma cells and scattered immunoblast-like cells
Immunoreactivity and molecular studies	• Standard reactive T and B cell markers • Polyclonal or Oligoclonal lymphocyte populations	• CD20 • CD79a • CD43 • t(14;18)(q21;q32) • Trisomy 3 • Trisomy 18 • Monoclonal by IgH Rearrangement • Monoclonal by Kappa/Lambda
Prognosis	• Non-curable condition • Most, if not all EMZBCL are preceded by LESA, necessitating appropriate patient follow up	• Indolent and localized disease • Transformation to higher grade large cell lymphoma may occur
Treatment	• Corticosteroids • Surgical excision	• Complete surgical excision • Parotidectomy • Radiation • Chemotherapy

Identifying the histopathologic changes that characterize the transition from LESA to EMZBCL has been diagnostically challenging due to their significant morphologic overlap (Table 2.). It has been proposed that EMZBCL arising in LESA originates from centrocyte-like marginal B cells encircling the LELs (Hyjek et al. 1988). Monoclonal expansion of these cells is thought to be the earliest indicator of lymphoma. Salivary gland EMZBCL, like that of other

sites, is an indolent disease until late in its course. This initial indolence, juxtaposed with the non-neoplastic nature of LESA, creates a dilemma in differentiating the malignant and benign processes (Hyjek et al. 1988). Because normal salivary gland does not contain extranodal lymphoid tissue, the development of LESA represents acquired mucosa-associated lymphoid tissue (MALT). MALT is the setting or milieu for the development of extranodal marginal zone B-cell lymphoma (Jaffe, 2002). LELs may be found in normal MALT, such as in intestinal and tonsillar tissues; however, LEL formation in acquired MALT is considered indicative of a developing lymphoproliferative disorder (Isaacson et al. 1999).

EMZBCL occurs most frequently in the seventh decade of life and rarely affects children or young adults (Ferry, 2008). Although EMZBCL is more common in the setting of SS, the presence of an underlying autoimmune disorder does not adversely affect the clinical outcome (Troch et al. 2011). This lymphoma is slow to disseminate and treatment by local excision and radiation therapy generally results in prolonged disease-free intervals. Transformation to diffuse large B-cell lymphoma is well-documented (Isaacson et al 2008; Abbondanzo, 2001) and mandates more aggressive treatment. Histologically, EMZBCL manifests as a diffuse or slightly nodular infiltrate of marginal zone B cells with irregular, reniform nuclei and clear cytoplasm (Ferry, 2008). Marginal zone B cells commonly overrun the reactive lymphoid follicles established in MALT, leaving scattered remnants of germinal centers and mantle B cells (Bacon et al. 2007). Although collections of monotypic plasma cells with Dutcher body formation may occur, this is a less common feature of salivary EMZBCL (Rawal et al. 2007). The LELs characteristic of LESA are also present in EMZBCL, except that there is an expansion of monocytoid marginal zone B cells (Sheibani et al. 1988) which demonstrate immunoglobulin

light chain restriction (Bacon et al. 2006). Invasion or destruction of the LELs by infiltrating monocytoid lymphocytes can be appreciated as well (Rawal et al. 2007).

Immunohistochemistry (IHC) is a technique applied to cases when the definitive diagnosis cannot be established on hematoxylin & eosin-stained slides alone (Jordan et al. 2002). There is no single immunohistochemical marker of EMZBCL (Bacon et al. 2006); diagnosis is therefore dependent on both morphological and immunophenotypic features (see Table 3). Marginal zone B cells react positively with CD20 and CD79a and are non-reactive for cyclin D1/BCL1, CD5 and CD10 (Ellis et al. 2001; Ferry, 2008; Troch et al. 2011). In 30-50% of cases, the neoplastic B cells express CD43 (Ellis et al. 2007; Ferry, 2008; Bacon et al 2006). The absence of BCL-2 expression in follicles supports a diagnosis of EMZBCL (Abbondanzo, 2001) over follicular lymphoma, although neoplastic B cells are typically BCL-2 positive.

Zulman et al. (1978) first reported monoclonality in SS-associated lymphomas by immunohistochemically demonstrating light chain restriction. The neoplastic B-cells were found to express exclusively kappa or lambda light chains indicating monoclonality. The distinction between LESA and EMZBCL may rest in identification of monoclonality in the neoplastic lesion, in contrast to the reactive, polyclonal lymphoid population of LESA (Diss et al. 1995). Normal human reactive B cell populations express kappa or lambda light chains within immunoglobulins in a 2:1 ratio respectively (Hyjek et al. 1978). Significant alteration in the normal kappa-lambda ratio is strong evidence of a monoclonal population of cells; predominance of one form of light chain is referred to as “light chain restriction” (Jordan et al. 2002).

Table 3: Immunophenotypic characteristics for EMZBCL		
IHC	***Cell Type***	***EMZBCL***
CD5(b)	All T cells and thymocytes(a) Expression in B cells is indicative of lymphoma	- (b)
CD10(b)	Immature B cells and some mature B cells(a)	- (b)
CD20(b)	B cells(a)	+ (b)
CD23(b,c)	Marker for small lymphocytic lymphoma(c)	- (b,c)
CD43(b)	Expression in B cells is indicative of lymphoma	50% + (b)
CD79a(b)	Mature B cells(a)	+ (b)
BCL2 (b)	Expression in marginal B cells is a indicative of lymphoma	+/- (b)
BCL1(b)	Expressed in mantle cell lymphoma(b)	- (b)

(a) Abbas AK, Lichtman AH, 2006
(b) Isaacson et al. 2008
(c) Abbondanzo, 2001

In 1995, Diss et al. reported on 45 paraffin-embedded parotid biopsy samples that were examined for monoclonality. Thirty one of the cases were originally diagnosed as LESA and 14 as MALT lymphoma. All monoclonal cases (thirty-six) and two polyclonal cases demonstrated expansion of monocytoid B-cell populations around LELs. Falzon et al. (1991) identified two patients who were diagnosed with LESA and subsequently developed extra-salivary gland lymphoma after 9-10 years. In both patients, the initial diagnosis of benign lymphoepithelial lesion was revised to MALT lymphoma once monoclonality was demonstrated in the form of light-chain restriction.

In these studies, the presence of monoclonality in a so-called LESA of salivary gland was interpreted as diagnostic of MALT lymphoma/EMZBCL (Falzon et al. 1991; Diss et al. 1995; Zulman et al. 1978). Although EMZBCL behaves in an indolent manner, the importance of diagnosing this lesion as a low-grade B cell lymphoma and managing as such can possibly ameliorate future morbidity if the disease disseminates or transforms into a more aggressive lymphoma (Falzon et al. 1991; Diss et al. 1995). Controversy exists regarding the significance of monoclonality in LESA. Some authors have suggested that monoclonality alone may not be a

reliable indicator of lymphoma (Abbondanzo, 2001) and have further proposed the possibility of multiple transitions between reactive and neoplastic status, in some circumstances because the clinical behavior is indolent (Carbone et al. 2000; Collins, 1997). There can be significant delays, as long as 29 years, between the first documentation of LESA and the development of frank lymphoma (Abbondanzo, 2001). The terms "monoclonal lymphoproliferative disease of undetermined significance (MLDUS)" and "clonal disorder of uncertain malignant potential" have been proposed for lymphoproliferative conditions that demonstrate a clonal population of lymphocytes (Collins, 1997), but do not behave as classical lymphoma.

Recently developed molecular techniques have helped identify genetic alterations that characterize EMZBCL. Fluorescence In Situ Hybridization (FISH) can be used to map genes and genetic translocations using fluorescent-tagged probes against the sequence of interest (Jordan et al. 2001). Approximately 25% of salivary gland EMZBCL harbor a t(14;18)(q32;q21) translocation, which results in the juxtaposition of the *IGH* and *MALT1* genes (Isaacson et al. 2008). Proximity to IgH, which encodes for immunoglobulin heavy chain, results in overexpression of *MALT1*, which encodes a caspase-like protein, (Bacon et al. 2007). Trisomy 3 is the most common genetic alteration of EMZBCL of parotid gland, followed by Trisomy 18 (Troch et al. 2011).

II. MATERIALS AND METHODS

A query was performed seeking samples with a diagnosis of “benign lymphoepithelial lesion”, “lymphoepithelial sialadenitis” or “myoepithelial sialadenitis”, diagnosed prior to 1991, utilizing the Joint Pathology Information Management System (JPIMS). The paraffin-embedded blocks and hematoxylin and eosin (H&E) stained slides for fifty cases meeting these criteria were retrieved from the Joint Pathology Center Tissue Repository (JPCTR) located at the Joint Pathology Center (JPC), Forest Glen Annex, Silver Spring, Maryland. Cases missing paraffin blocks or demonstrating insufficient remaining tissue for testing were immediately eliminated. . In the interest of protecting personally identifiable information, a new identification number was created for each case and recorded on a separately-maintained master list with the corresponding JPIMS accession number. The cases were then reviewed in conference by the associate investigators (AIs) and the first twenty cases with the correct diagnosis and sufficient paraffin-embedded tissue were selected for the study. When available, the pertinent demographic and medical information was recorded for each case on the data collection sheet (Table 4).

Table 4: Data Collection			
Specimen#_____			
Age			
Gender			
Ethnicity			
Medications			
Medical History			
Tissue Site			
Part I: Immunohistochemistry for Characterization			
	Present	Absent	Comments (indicate if undetermined here)
CD5			
CD10			
CD20			
CD23			
CD43			
CD79a			
Cyclin D1			
BCL-2			
PAX-5			
Part II: Light Chain Restriction			
	Present (%)	Absent	Comments (indicate if undetermined here)
Kappa			
Lambda			
Part III: Ig Heavy Chain Rearrangement			
Circle one: Monoclonal Polyclonal Oligoclonal Indeterminate Comments:			
Part IV: Cytogenetic Abnormalities			
Circle one: Observed Not observed Undetermined Comments:			

Immunohistochemistry. A Roche Ventana Benchmark Ultra automated slide-stainer was utilized for application of the IHCs. Formalin-fixed, paraffin-embedded tissue samples were sectioned at 4µm, applied to positive-charge slides, deparaffinized in xylene, rehydrated through sequential passes through 100%, 95% and 80% ethanol, and equilibrated in a 7.6 pH tris-based buffer. The slides were heated to 37ºC and incubated with pre-diluted antibody (see Table 4) for 32 minutes (48 minutes for CD5) and counterstained with hematoxylin with subsequent application of bluing reagent.

Table 5: Immunohistochemical antibody panel

Antibody	***Clone (a)***	***Source (b)***	***Antigen retrieval (c)***
CD5	SP19	Ventana	Water bath
CD10	56C6	Ventana	Water bath
CD20	L26	Ventana	Water bath
CD23	SP23	Ventana	Water bath
CD43	L60	Ventana	Water bath
CD79a	SP18	Ventana	Water bath
Kappa	Polyclonal	Ventana	Water bath
Lambda	Polyclonal	Ventana	Water bath
Cyclin D1/BCL1	SP4-R	Ventana	Water bath
BCL-2	124	Ventana	Water bath
PAX-5	SP34	Ventana	Water bath

(a) All antibodies are monoclonal unless otherwise specified
(b) Ventana, Ventana Medical Systems, Tucson AZ
(c) Slides are heated at 95ºC in a tris-based buffer (Ventana cell conditioning solution CC1) for 8 minutes

Ig Heavy Chain Gene Rearrangement. Immunoglobulin heavy chain gene rearrangement studies were performed via polymerase chain reaction (PCR) using formalin-fixed, paraffin-embedded tissue as described by Reed et al. (1993): Two 7-8 µm paraffin sections from each block were deparaffinized in xylene, rehydrated with ethanol, and dried. Samples were then digested with Proteinase K at 55ºC for 1 hour, followed by heating to 95ºC for 10 minutes. The samples were centrifuged and placed into a thermal cycler using approximately 1 U *Taq*

polymerase, 100 nM of each primer (see Table 6), 200 µM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM tris-HCl, and .01% gelatin. The semi-nested PCR approach was performed as specified in Wan et al. 1990. In a semi-nested PCR protocol, one of the primers used in the second set of cycles hybridizes to a sequence within the corresponding primer in the first set, such that nonspecific amplification products of the first cycles (so-called outer nest) are not amplified in the second cycles (inner nest) (Reed et al. 1993).

Table 6: Primers used for PCR(a)

Primer	***Sequence 5'-3'***
V region (FR3A)	ACA-CGG-C(C/T)(G/C)-TGT-ATT-ACT-GT
J Region (CFW1; outer nest)	ACC-TGA-GGA-GAC-GGT-GAC-CAG-GGT
J Region (VLJH; inner nest)	GTG-ACC-AGG-GT(A/G/C/T)-CCT-TGG-CCC-CAG

(a) Reed et al. 1993; Wan et al. 1990.

The initial amplification was performed with primers FR3A and CFW1 as follows:

- 5 minutes of denaturation at 94ºC
- 40 cycles of: 1-minute denaturation at 94ºC, 1-minute annealing at 55ºC, 1-minute chain extension at 72ºC
- 7 minutes of chain elongation at 72ºC

For the next step, 10µl of a 1:1,000 dilution from each tube was transferred to a new tube with fresh reagents (including fluorescent tags) with FR3A and VLJH primers:

- 5 minutes of denaturation at 94ºC
- 20 cycles of: 1-minute denaturation at 94ºC, 1-minute annealing at 55ºC, 1-minute chain extension at 72ºC
- 7 minutes of chain elongation at 72ºC

Amplified products were separated by capillary electrophoresis utilizing an ABI 3100 (Applied Biosystems, Foster City, CA). Samples were electrophoretically injected into thin, fused-silica capillaries that have been filled with polymer. A voltage was applied causing DNA fragments to migrate towards the (+) end of the capillaries, with shorter fragments moving faster than longer fragments. The fragments fluoresce as they move through an excitation beam and this information is captured by an optical device and transferred to a computer workstation for processing.

Fluorescence* in situ *Hybridization. EMZBCL translocations involving an 18q21.31 (MALT1 gene) rearrangement were identified utilizing the Vysis LSI ® MALT1 Break Apart FISH Probe Kit (Abbott Molecular, Downer Grove, IL) (figure 1).

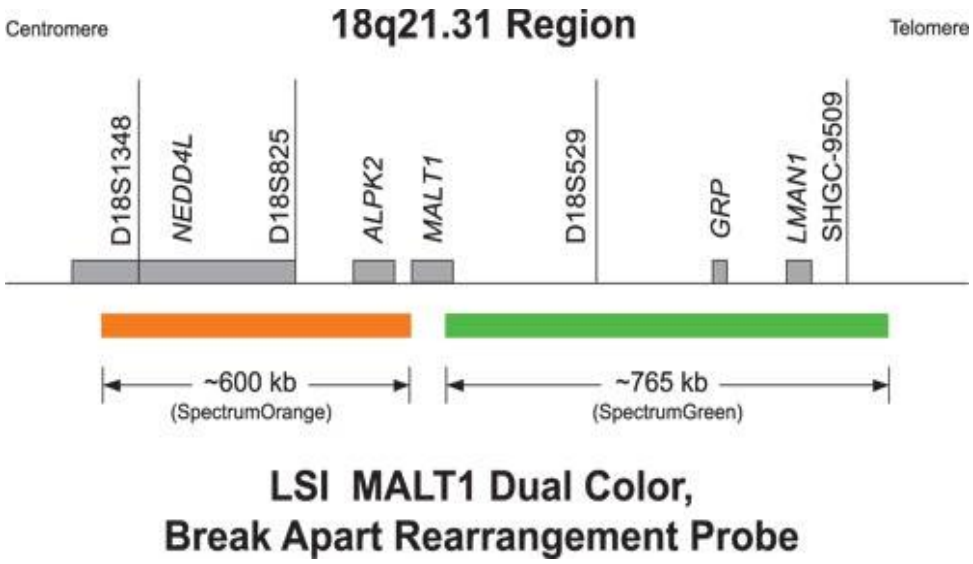

Figure 1: The Vysis LSI MALT1 break apart probe is used as a screening mechanism to identify the presence of translocations involving the *MALT1* gene on chromosome 18. "Vysis LSI MALT1 Dual Color Break Apart Rearrangement Probe." . Abbott Molecular, n.d. Web. 6 May 2014. <https://www.abbottmolecular.com/us/products /analyte-specific-reagent/fish/vysis-lsi-malt1-18q21-dual-color-break-apart-rearrangement-probe.html>.

Four µm formalin fixed, paraffin embedded sections were first incubated for 24 hours in a 56°C oven and then deparaffinized in Hemo De® (xylene-alternative solvent) and dehydrated in 100% ethanol before being immersed in a pre-treatment solution (HCl 0.2N) for 25 minutes in a 97°C water bath. After washing with deionized water (dH_2O), the slides were immersed in protease (60mg/ml pepsin) and protease buffer for 45 minutes in a 37ºC water bath to facilitate DNA access for the probes. The slides were then placed in wash buffer for 5 minutes and rinsed in dH_2O. Approximately 10 µl of probe mix (7 µl locus-specific identifier (LSI) buffer, 1 µl LSI DNA probe, 2 µl purified water) was applied to each specimen, covered with coverslip and sealed with rubber cement. (Note: one probe is labeled with SpectrumOrange and is specific for *ALPK2* [~600 kb], while the second probe is labeled with SpectrumGreen and is specific for *MALT1* [~765 kb]). The slides were incubated in a Thermobrite Statspin® (Abbott Molecular, Downer Grove, IL) slide incubator at 75ºC for 6 minutes followed by 37ºC for 24 hours to automate the denaturation and hybridization steps.

After the rubber cement was removed, slides were placed in post-hybridization buffer I at room temperature to soak off the coverslips. Slides were then placed in a Coplin jars containing post hybridization buffer I pre-heated to 75°C in a water bath for 5 minutes. Slides were then washed in post-hybridization buffer II for 30 seconds at room temperature, rinsed in dH_2O, counterstained with 10 µl DAPI (4',6-diamidino-2-phenylindole) blue fluorescent stain and coverslipped. Slides were then placed in -20°C for 30 minutes and reviewed using Bioview Ltd. Cytogenetics Suite software and a fluorescent microscope. This procedure was repeated twice more using chromosome enumeration probes (CEP) in SpectrumOrange targeted at the

centromeres of chromosomes 3 and 18 (CEP3, 3p11.1-q11.1 Alpha Satellite DNA; CEP18, 18p11.1-q11.1 Alpha Satellite DNA), also provided by Vysis (figure 2).

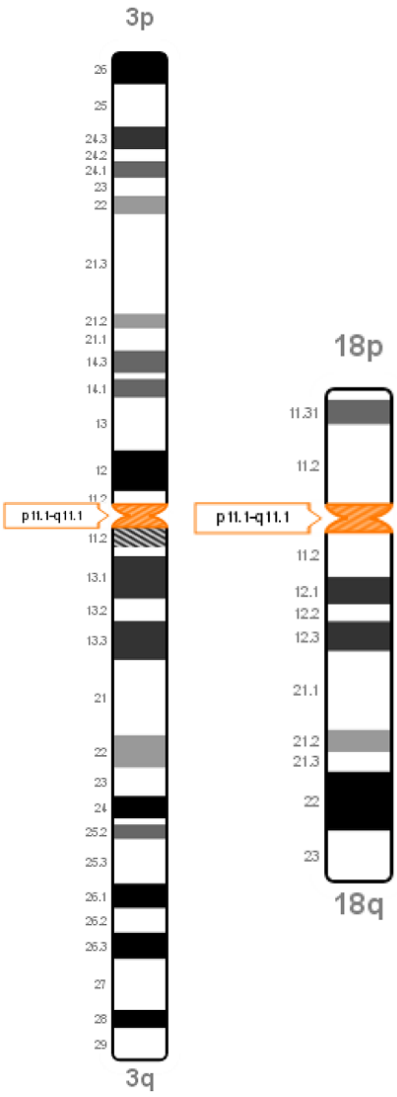

Figure 2: The Vysis CEP probes for chromosomes 3 and 18 allow the identification and counting of the appropriate chromosome by targeting the centromere. "Vysis CEP Probes." Abbott Molecular, n.d. Web. 6 May 2014. <https://www.abbottmolecular.com/us/products/analyte-specific-reagents/Centromere-CEP-probes.html>.

III. RESULTS

The patient population comprised 19 females and 1 male, ages 16 to 78 years, with a mean age of 52.6 and a median age of 47. The available medical histories are reported in table 7. Five patients had a reported history of autoimmune diseases, which included Sjögren syndrome, inflammatory polyarthralgia, rheumatoid arthritis and Raynaud phenomenon.

Table 7: Patient Information

Case	*Age*	*Gender*	*Ethnicity*	*Medical History*
1	49	F	Caucasian	NR
2	60	F	Caucasian	NR
7	61	F	Caucasian	Large cell lymphoma of GI tract
8	78	F	Caucasian	NR
11	58	F	Caucasian	NR
12	63	F	Caucasian	NR
15	73	F	Caucasian	NR
16	35	F	Caucasian	Raynaud syndrome
20	68	F	Caucasian	Sjögren syndrome Breast malignancy
24	47	F	Caucasian	Rheumatoid arthritis
26	45	F	Caucasian	NR
28	16	F	Caucasian	Bilateral cervical lymph nodes
30	59	F	NR	NR
31	48	M	NR	NR
39	NR	F	NR	Sjögren Syndrome
43	30	F	NR	NR
46	24	F	NR	Inflammatory polyarthralgia Rheumatoid arthritis
48	65	F	American Indian	Hypothyroidism
49	64	F	NR	NR
50	57	F	Caucasian	No auto-immune disease

NR=Not reported

The youngest patient, a 16 year old female, presented with bilateral cervical lymphadenopathy but no additional information was provided and no follow up materials were available. Malignancies reported included a non-specific breast cancer and large cell lymphoma of the ileum.

The morphology of each specimen was assessed by evaluating for salient features which may distinguish LESA from EMZBCL; Table 8 summarizes the morphologic findings. The specimens demonstrated variable effacement by lymphoid tissue with corresponding residual parenchyma intermixed or relegated to the periphery (Figure 3).

Table 8: Salient Morphologic Features

Morphology	***Present***	***Absent***	***Percetange present***
Halos Present	20	0	100
Halos Coalescing	15	5	75
Follicles Atrophic	12	8	60
Follicles Serpeginous	7	13	35
Lobules Expanded	13	7	65
Lobules Effaced	7	13	35
Residual Parenchyma	14	6	70

All specimens demonstrated lymphoepithelial lesions (Figure 4), a key feature of both LESA and EMZBCL. The presence of monocytoid halos around these lymphoepithelial lesions was identified in all specimens as well, although in only 15 cases was there coalescing or sheeting of these halos, a finding more consistent with EMZBCL (Quintana et al, 1997).

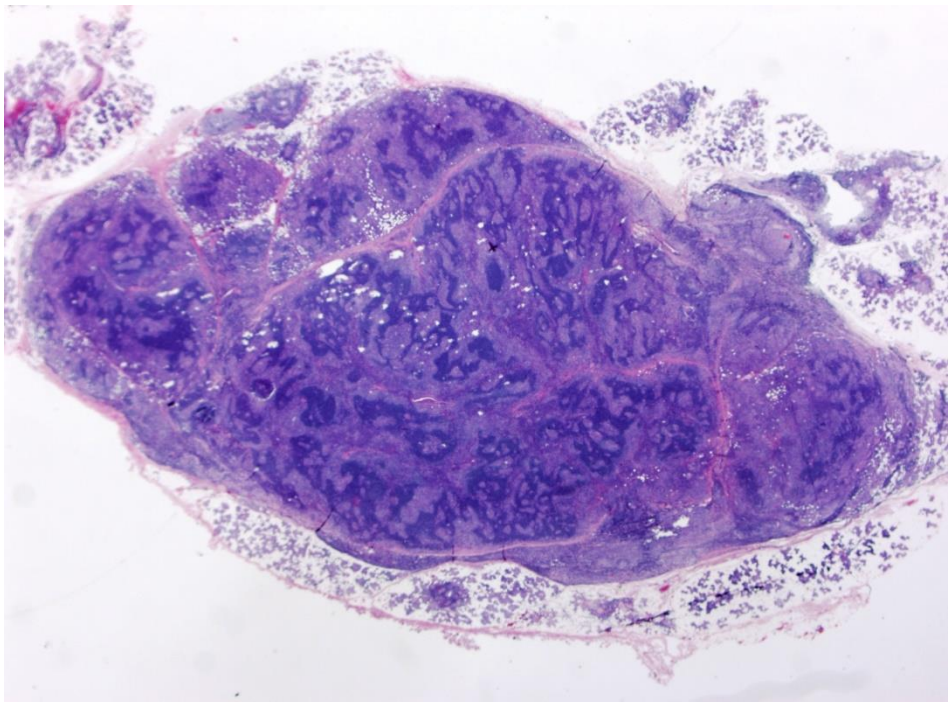

Figure 3: Scanning power view of an excised portion of parotid gland tissue demonstrates effacement by lymphoid tissue with expanded yet preserved lobular architecture. Residual parenchyma is relegated to the periphery.

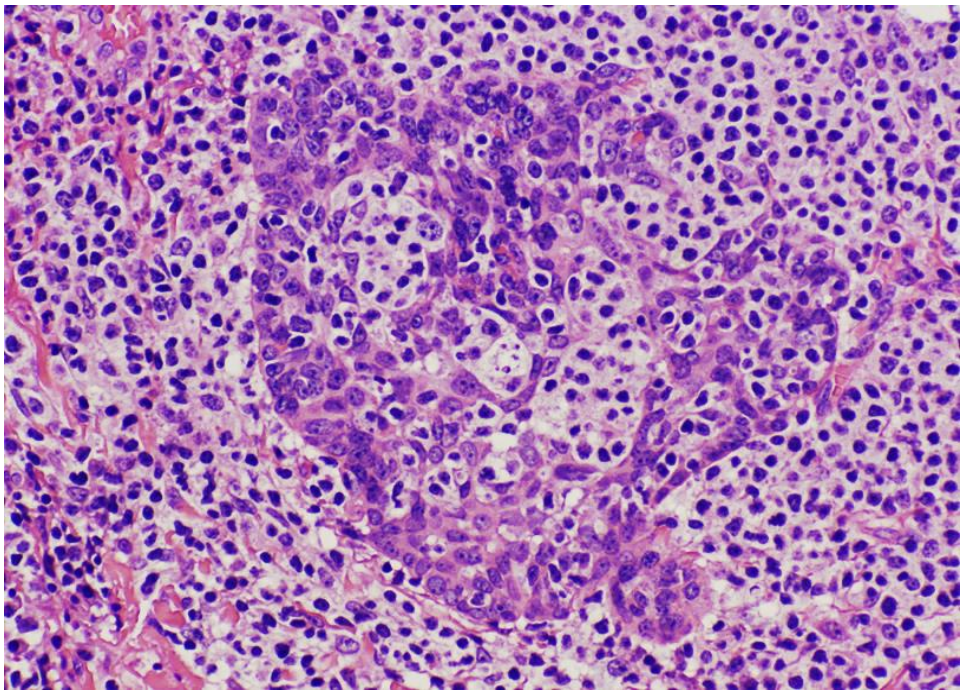

Figure 4: Lymphoepithelial lesion formation characterized by distortion and metaplasia due to ifiltrative monocytoid B cells. A halo of these cells is present in the periphery.

The epitheliotrophic lymphocytes demonstrated reactivity for CD20, CD79a and PAX5, consistent with a B cell immunophenotype (Figure 3). In 17 cases, these B cells were CD43 positive (Table 9, Figure 4) which is considered abberant and suggestive of a neoplastic proliferative process. Of these cases, 2 were also CD5 positive, a finding considered rare for EMZBCL but nonetheless documented in the literature (Jaso et al, 2011). Diagnoses of mantle cell lymphoma and chronic lymphocytic leukemia/small lymphocytic lymphoma were ruled out with negative reactivity for BCL1/CyclinD1 and CD23, respectively (Figure 5). In most cases, CD10 demonstrated a combination of atrophic and expanded germinal centers with many of the original residual follicular dendritic meshworks (visible with CD23) not corresponding the aforementioned CD10 positive germinal centers (Figure 6). Case 49 did not react for any immunostains due to fixative issues (the specimen was fixed in Lillie's solution).

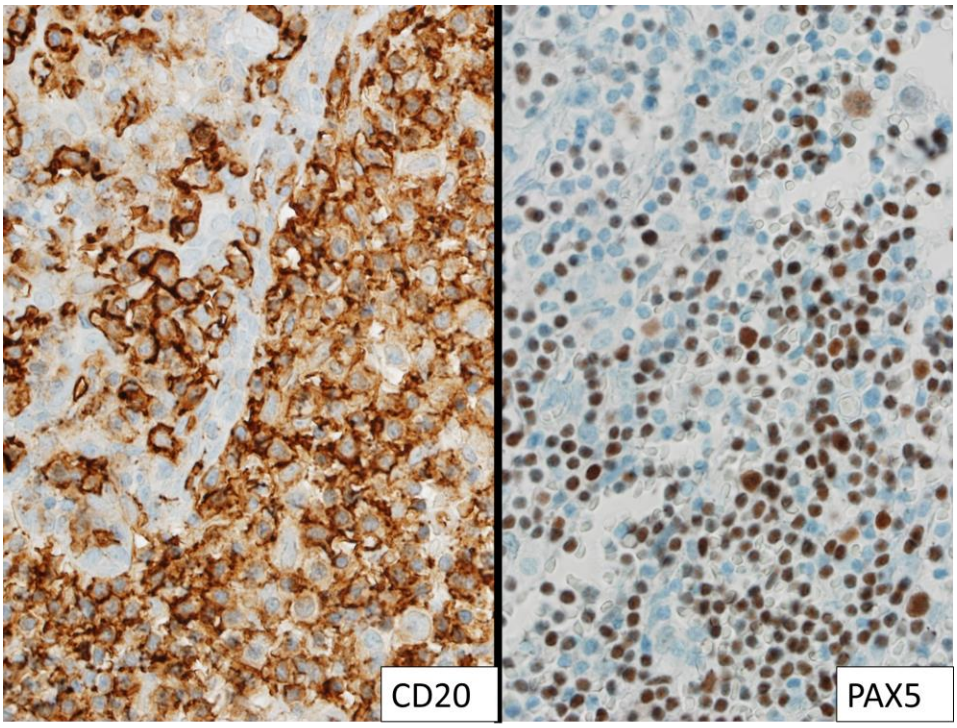

Figure 5: The epitheliotrophic lymphocytes demonstrate a B cell immunophenotype. PAX-5 highlights mitotic figures.

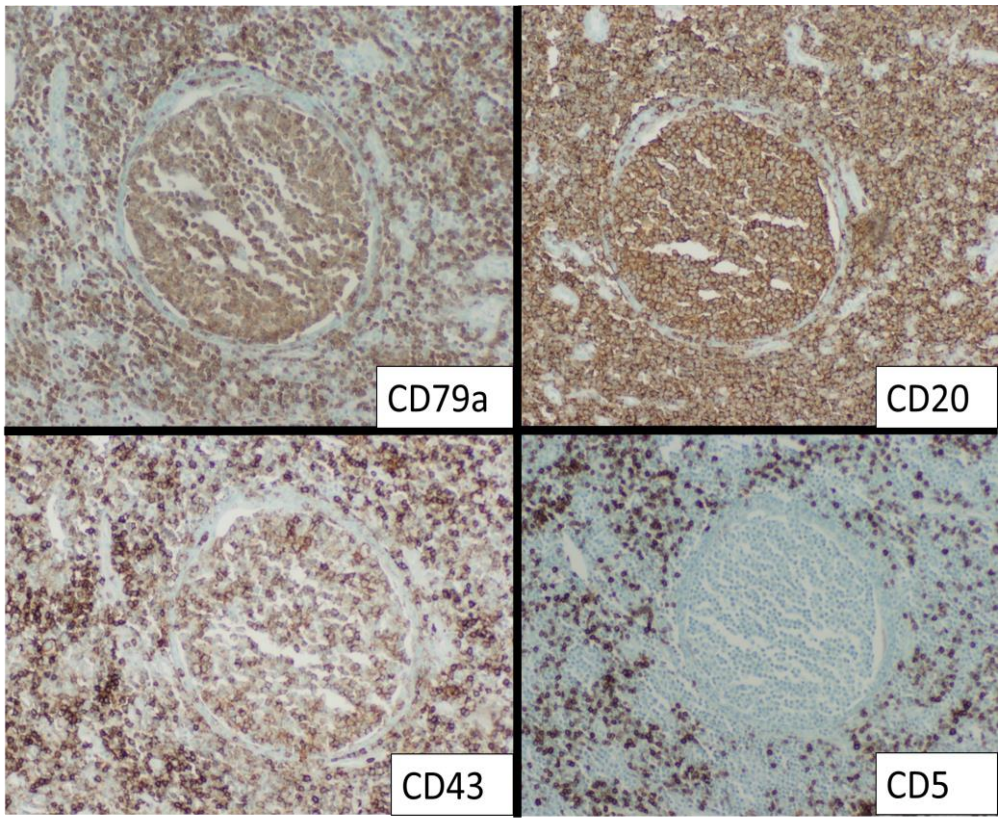

Figure 6: Aberrant CD43 reactivity in B cells is suggestive of lymphoma.

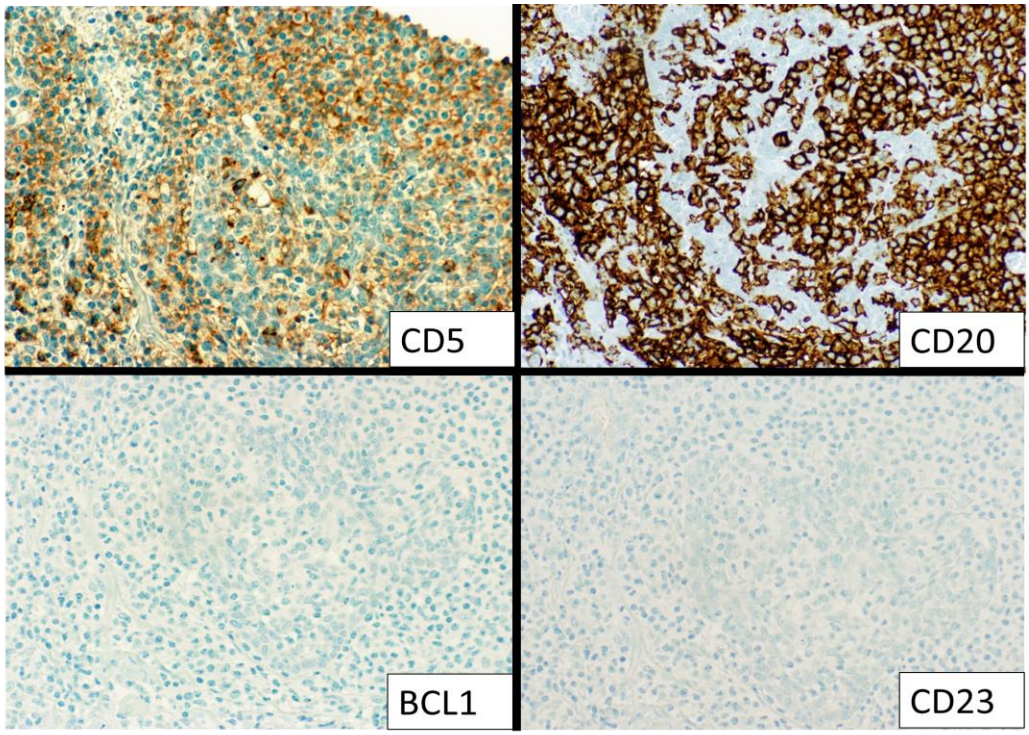

Figure 7: One of two cases which demonstrated CD5 reactivity; a lack of staining with BCL1 and CD23 rules out other small B cell lymphomas.

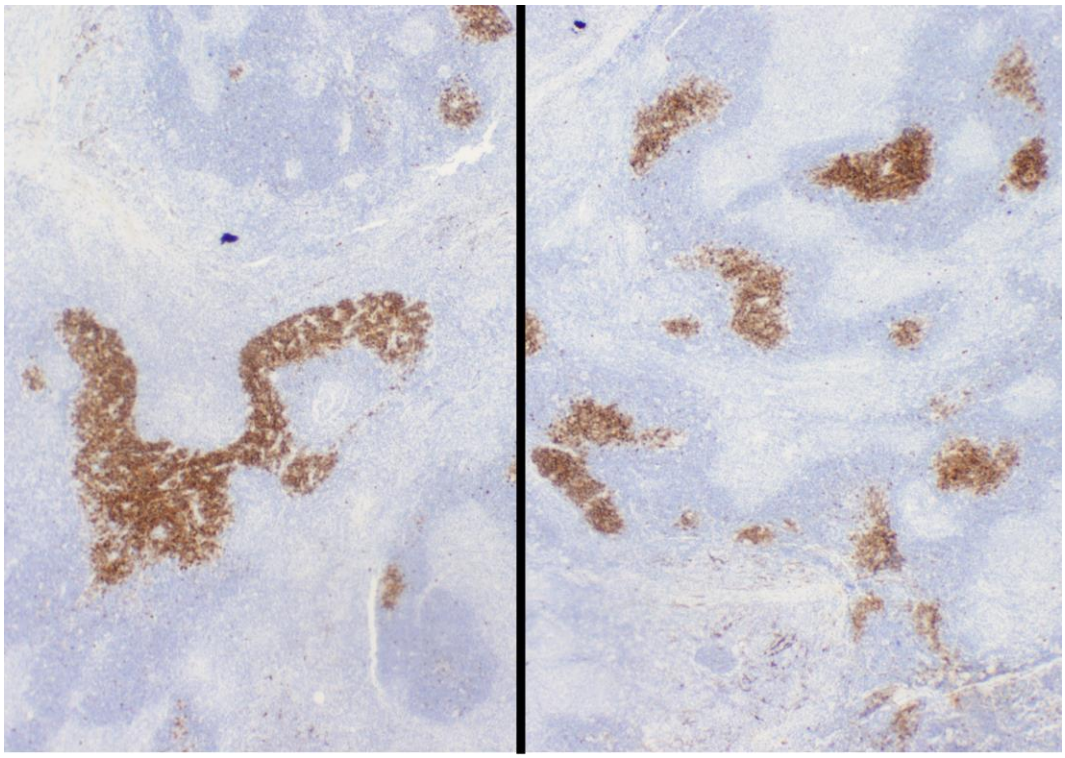

Figure 8: Germinal centers were highlighted by CD10 and varied from expanded and serpingenous (left) to atrophic (right)

Table 9: Results of Molecular Testing							
#	**Location**	**CD43**	**Light Chain**	**IgH**	**MALT1**	**CEP 18**	**CEP 3**
1	Parotid, Right	Positive	Restricted	Monoclonal	Indeterminate	Normal	Increased
2	Parotid, Right	Positive	Restricted	Monoclonal	Negative, increased signals	Increased	Normal
7	Parotid, Left	Positive	Restricted	Negative	Negative	Increased	Increased
8	Parotid, Right	Positive	Restricted	Monoclonal	Negative	Increased	Increased
11	Parotid, NOS	Positive	Restricted	Monoclonal	Negative, increased signals	Increased	Increased
12	Parotid, Left	Positive	Restricted	Monoclonal	Negative, increased signals	Increased	Normal
15	Parotid, Left	Positive	Restricted	Monoclonal	Negative, increased signals	Increased	Increased
16	Parotid, Right	Positive	Restricted	Negative	Negative	Normal	Normal
20	Parotid, Right	Positive	Restricted	Monoclonal	Negative	Increased	Increased
24	Submandibular, Right	Positive	Restricted	Monoclonal	Negative, increased signals	Increased	Normal
26	Parotid, Right	Positive	Restricted	Monoclonal	Negative	Normal	Normal
28	Parotid, Right	Positive	Restricted	Oligoclonal	Negative	Normal	Normal
30	Submandibular, Left	Positive	Restricted	Monoclonal	Negative, increased signals	Normal	Normal
31	Parotid, Right	Positive	Restricted	Monoclonal	Negative, increased signals	Increased	Increased
39	Parotid, Right	Positive	Unrestricted	Monoclonal	Negative	Normal	Normal
43	Parotid, Left	Positive	Restricted	Monoclonal	Negative	Normal	Increased
46	Parotid, Right	Positive	Restricted	Monoclonal	Negative	Normal	Indeterminate
48	Parotid, Left	Positive	Restricted	Monoclonal	Negative	Increased	Normal
49	Parotid, Left	Failed	Failed	Monoclonal	Negative, increased signals	Increased	Normal
50	Parotid, Left	Negative	Unrestricted	Indeterminate	Indeterminate	Indeterminate	Indeterminate

Seventeen cases were light chain restricted, specifically kappa light chain restricted. This finding is somewhat expected in that many B cell neoplasms demonstrate a preference for kappa restriction as opposed to lambda. Fifteen cases demonstrated monoclonality of the Ig heavy chain rearrangement by PCR and capillary electrophoresis, and one case was oligoclonal. The oligoclonal case involved a 16 year old female with right parotid gland involvement and bilateral cervical lymphadenopathy at presentation. This case demonstrated light chain restriction and CD43 positivity.

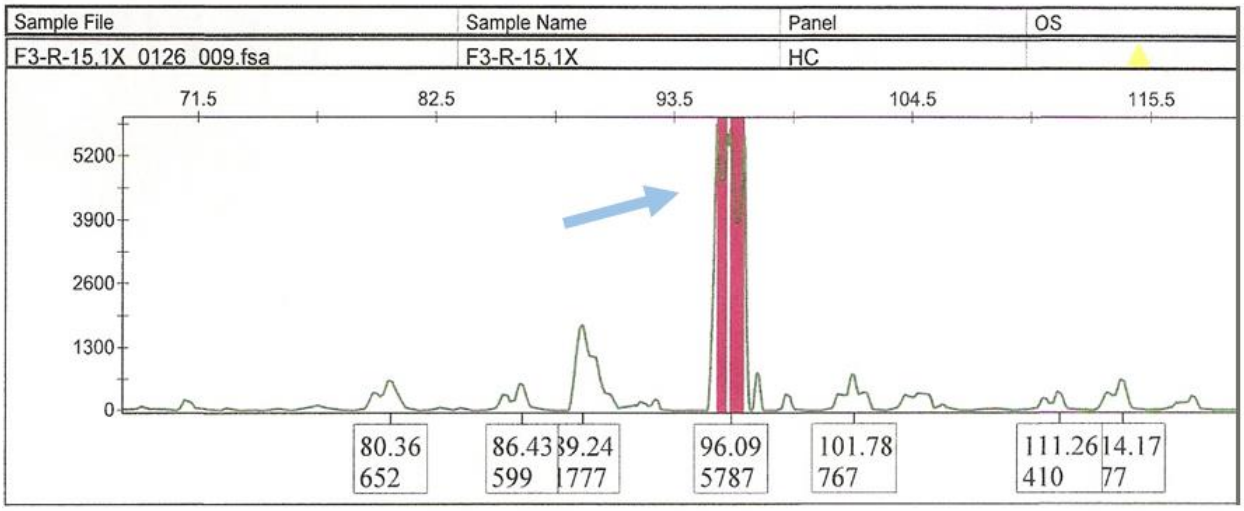

Figure 9: Capillary electrophoresis sorts PCR products by size; a peak indicates a population of PCR products of the same size,

All monoclonal cases demonstrated a peak in the Frame 3 region of the rearranged segment, which is not unexpected, as Frame 3 is the shortest. Only one case (case 50) was indeterminate, likely due to fixative issues affecting the DNA quality

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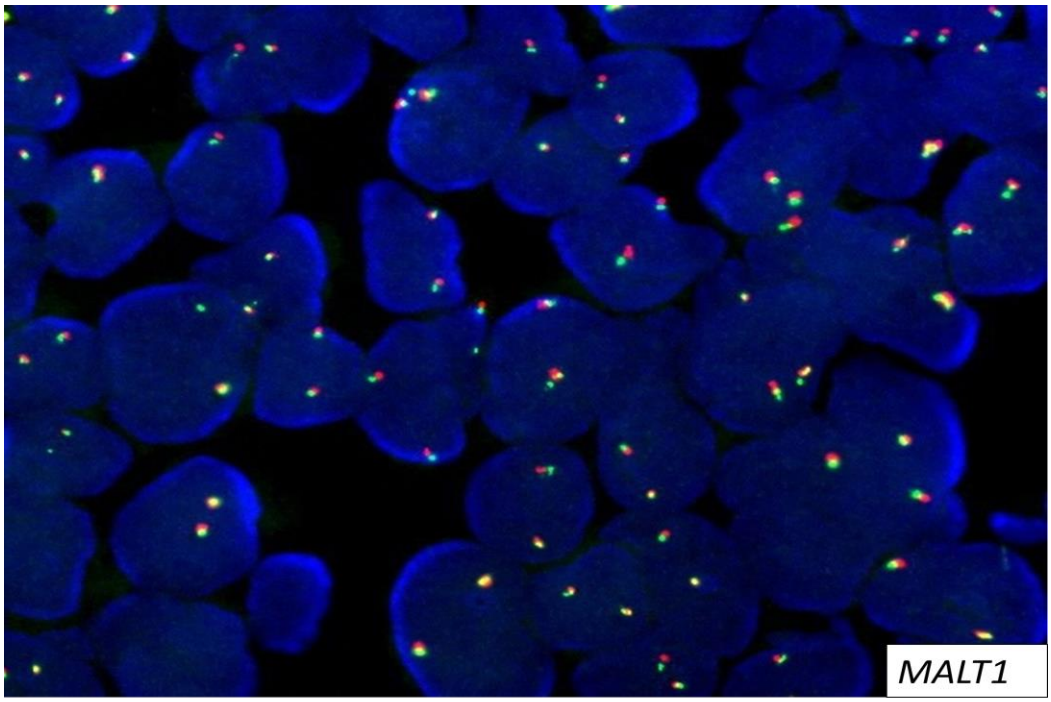

Figure 10: *MALT1* breakapart probe evaluated by fluorescence *in situ* hybridization demonstrates two intact breakapart probes per cell which suggests there are no translocations involving this gene.

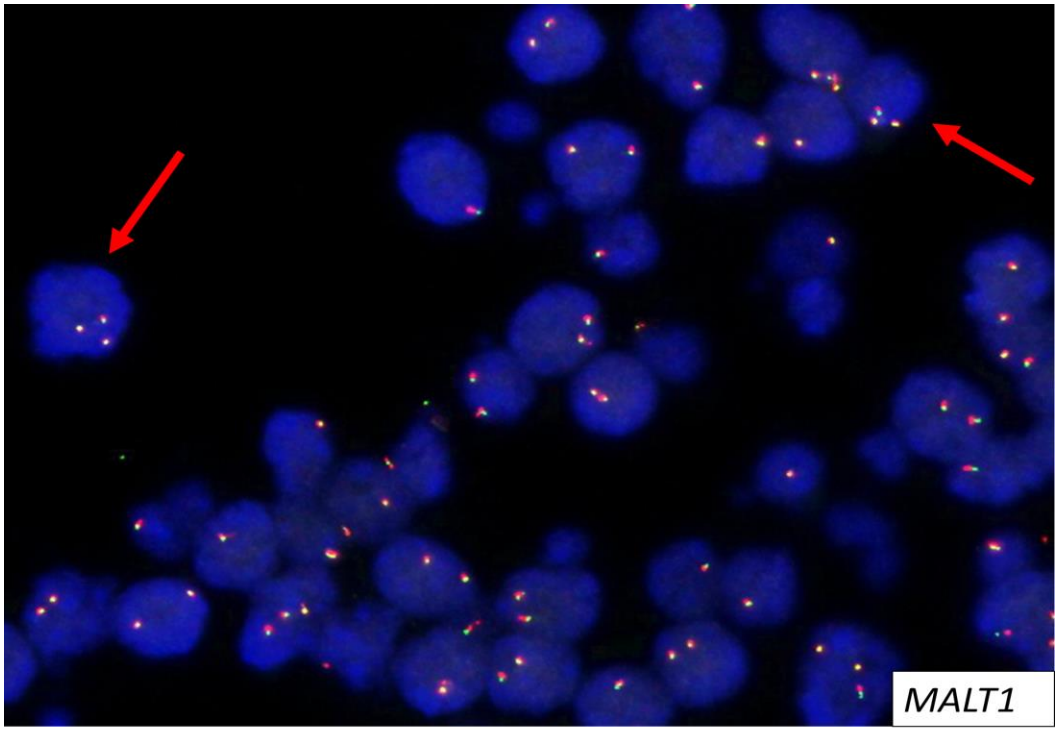

Figure 11: Many cases revealed multiple intact *MALT1* breakapart probes which suggested an increased copy number of chromosome 18.

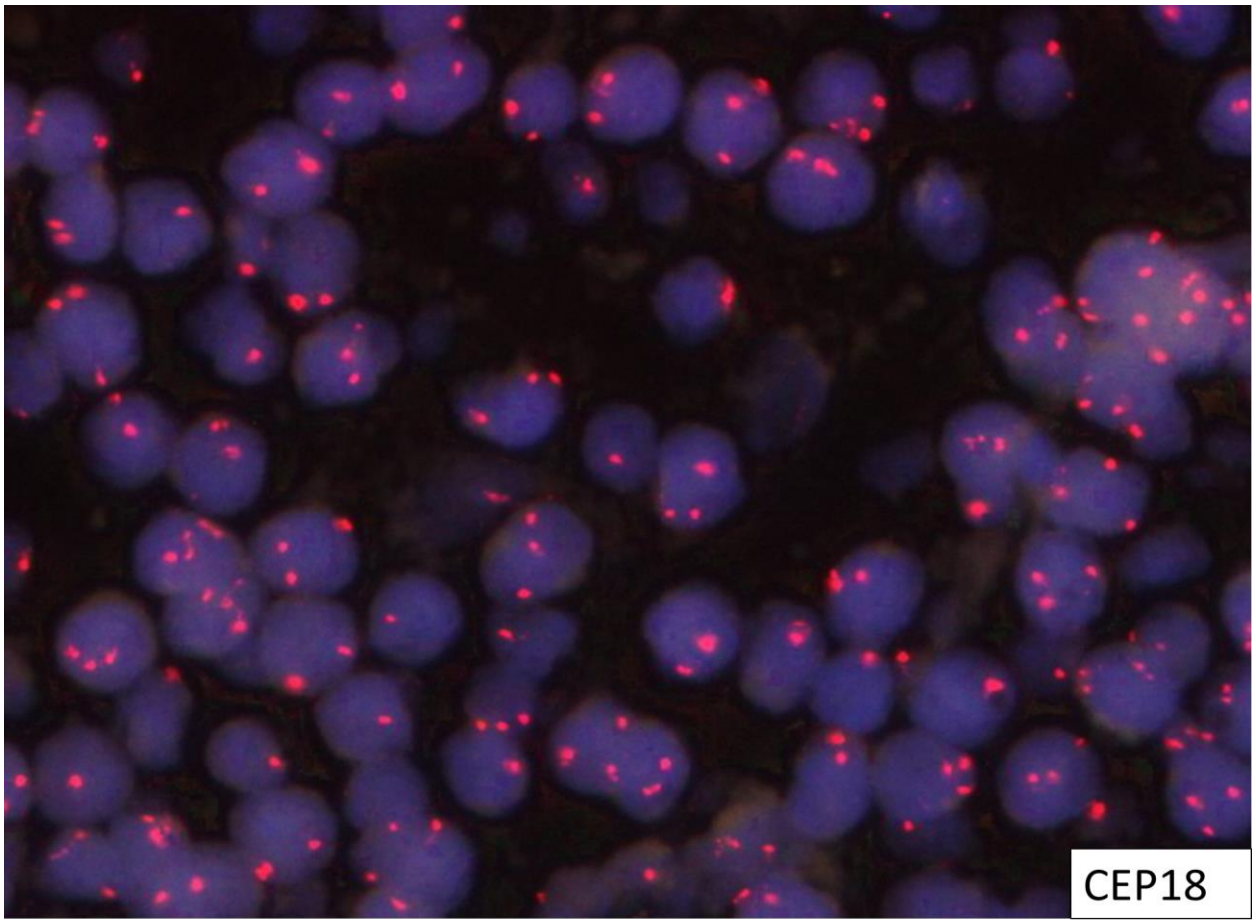

Figure 12: Chromosome enumeration probe (CEP) 18 was used to count the copy number of chromosome 18 to verify *MALT1* breakapart probe findings of increased copy number. Numerous cells here demonstrate multiple signals which represents increased copy number.

Although no translocations involving the *MALT1* gene were present, 8 cases showed increased copy number of intact breakapart probes (Figure 8). This finding was confirmed using the CEP18 probe and revealed an additional 3 cases with increased copy number of chromosome 18. Eight cases demonstrated increased copy number of chromosome 3 and a total of 6 cases had increased copy number of both chromosomes. Due to fixative issues, FISH results on one case (case 50) were unable to be determined.

IV. DISCUSSION

The diagnosis of lymphoma requires the confirmation of B lymphocyte monoclonality with or without aberrant immunohistochemical reactivity and a distinct architectural morphology (Fend et al, 2013). In all cases reviewed the presence of lymphoepithelial lesions with monocytoid halos was identified, a finding consistent with EMZBCL. All cases demonstrated monoclonality by either light chain restriction, heavy chain rearrangement, or both. Of interest, however, were five cases which had halos of monocytoid B cells which did not coalesce or sheet out, yet still demonstrated monoclonality. The presence of halos are considered the "emergence" of EMZBCL in salivary gland (Bacon et al, 2006) and considered the morphological checkpoint at which monoclonality may first be detected (Diss et al, 1995), however, the mere presence of B lymphocytes surrounding and infiltrating epithelial ducts is also considered suggestive of LESA (Jaffe et al, 2002). The morphologic distinction between LESA and EMZBCL, therefore, appears to depend on, among other features, the relatively gray area of B lymphocytes surrounding and infiltrating the duct, especially in the setting of B cell monoclonality. An extensive review of the literature failed to define what exactly constituted a "halo" of monocytoid B cells other than the presence of such B cells around an epithelial structure, which as previously mentioned, may also be seen in LESA, but to a lesser extent. In our findings, those five cases without coalescent monocytoid cell halos demonstrated either aberrant CD43 reactivity, BCL-2 reactivity (typical reactive monocytoid B cells are BCL-2 negative) or cytogenetic abnormalities seen in EMZBCL.

Monoclonality of B lymphocytes in LESA is considered a common finding in patients with Sjögren syndrome and is not necessarily indicative of lymphoma (Rodgriques et al, 2013), but

certainly precedes and may be an important change leading up to malignant transformation. Lymphoma in patients with auto-immune disease can therefore be considered a continuous spectrum of disease, vice an all or nothing phenomenon, which may explain the high rate of lymphoma in patients with Sjögren syndrome (Youinou et al, 2010).

Seventeen cases demonstrated aberrant CD43 reactivity in epitheliotropic B lymphocytes which is suggestive of lymphoma, but does not indicate whether a lymphoproliferative process confined to the salivary gland will disseminate (Hsi et al, 1995). The finding of CD5 reactivity is unusual but documented (Jaso et al, 2012) and is more associated with nongastric EMZBCL. Unlike CD43, CD5 appears to be indicative of disease dissemination, however, overall survival is excellent with the administration of appropriate therapy (Ferry et al, 1996).

The most common cytogenetic abnormality was increased copy number of chromosome 18, which has been documented in the literature as being the second most common (trisomy 18) next to trisomy 3 (Troch et al, 2011). The likely mechanism involves increased transcription of the *MALT1* gene which is a key oncogene involved in the tumorigenesis of EMZBCL in parotid gland. Of interest is the significance of trisomy 3 in EMZBCL. The high incidence is well-documented in the literature and in direct contrast to that seen in nodal marginal zone lymphoma (Wotherspoon et al, 1995). The underlying genetic mechanism is unknown but speculated to be associated with BCL-6 which is located at 3p27 (Wotherspoon et al, 1995).

Although EMZBCL is considered indolent, a high relapse rate has been associated with it which warrants appropriate diagnosis, treatment and follow-up (Raderer et al, 2005). In our

series, the findings of neoplastic features would warrant the classification of lymphoma in each of the cases were they diagnosed in modern day. We speculate that these patients were likely treated by the excision they underwent at time of diagnosis, lived with the disease until expiring of other causes, or experienced dissemination and sought care as a result.

In conclusion, the findings of neoplastic features in what were previously classified as reactive inflammatory lesions warrants re-evaluation of morphologic features existing in both EMZBCL and LESA due to more sophisticated molecular testing and immunohistochemistry. The findings of cytogenetic abnormalities and aberrant reactivity in lesions which morphologically appear benign is significant to our understanding of neoplasia and malignancy, and should encourage further studies to re-define the morphologic spectrum between LESA and EMZBCL.

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